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c-Myc oncogene has been reported to be amplified in 25-30% and overexpressed in more than 70% of human breast cancers and has been demonstrated to be involved in signaling cell proliferation and apoptosis. The Bcl-xL protein, a member of the Bcl-2 apoptosis-modulatory family, is known to block apoptotic cell death under a wide variety of conditions and has been shown to be overexpressed in some human breast cancers and breast cancer cell lines. Evidence from a c-Myc/TGFa bitransgenic mouse model suggests that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression and neoplastic development. The focus of this study is to determine if Bcl-xL expression cooperates with c-Myc overexpression in mammary tumorigenesis in vivo and in vitro by blocking c-Myc-induced apoptosis but not c-Myc-mediated cell cycle progression and cell proliferation.

Transgenic c-Myc mice will be mated with transgenic Bcl-xL mice to produce bitransgenic offspring which will be observed to determine whether Bcl-xL expression is sufficient to synergize with c-Myc and enhance mammary tumor incidence and accelerate tumor onset (latency). The utilization of this model system will aid in the dissection of the molecular mechanisms behind the development of breast cancer.

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Matt Hufman 28 August 1998

PI - Signature Date

MH INITIALS

TABLE OF CONTENTS

for Annual Report Grant # DAMD17-97-1-7110
"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"
P.I. Matthew Hunter Jamerson

This Annual Report addresses Grant # DAMD17-97-1-7110, a Pre-Doctoral Training Fellowship covering research conducted by the principal investigator Matthew Hunter Jamerson (an M.D./Ph.D. student at the Lombardi Cancer Center, Georgetown University Medical Center), entitled "Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis."

INTRODUCTION:

It is commonly held that the c-Myc oncogene is involved in the initiation and progression of human breast cancers due, in part, to the fact that it is found to be amplified in 25-30% of human breast cancers and is overexpressed in the absence of amplification in over 70% of human breast cancers (1,2). c-Myc oncogene is known to subserve cell cycle progression and cellular transformation as well as inhibit cellular differentiation and promote apoptosis under certain circumstances (3,4). Furthermore, deregulated c-Myc expression is common in human cancers and may contribute to cellular proliferation only when apoptosis is blocked (5,6,7,8). c-Myc is believed to be one of the nuclear mediators of mitogenic signals incident upon cells from various receptor systems and is contributory to but not sufficient for mammary epithelial cell (MEC) transformation (1,2,9).

c-Myc is a nuclear transcription factor that is known to be capable of interacting with DNA when heterodimerized with the Max protein (required for c-Myc-mediated transformation, cell cycle progression, and apoptosis) via leucine zipper and helix-loop-helix motifs (3,4,10). Deregulated c-Myc expression has been shown to partially transform mouse mammary epithelial cells (MMECs) such that they can be grown on soft agar (anchorage-independent growth) and are no longer as dependent upon growth factors as the parental, non-transfected cells (1). Additionally, c-Myc expression coupled with any block to cellular proliferation (such as growth arrest caused by serum deprivation) has been demonstrated in fibroblasts to result in apoptosis independent of cell cycle phase (5).

Bcl-x_L, a member of the Bcl-2 apoptosis-modulatory family of proteins, is the one of two related proteins produced from differentially spliced mRNAs (the other is the death inductive Bcl-x_S) that has been demonstrated to bind Bcl-x_S, Bax, Bad, and Bak (11,12,13,14,15). Bcl-x_L protein has been shown to block apoptosis induced by p53 in the T47D breast cancer cell line (16), by TNF and anti-FAS in the MCF-7 breast cancer cell line (17), and by serum deprivation, cytotoxic chemotherapeutic treatment, or γ-irradiation in other cell systems (18,19). Bad protein binds with high affinity to Bcl-x_L, unless phosphorylated by the Akt kinase in response to survival signaling via growth factor or cytokine receptors, and displaces Bax from heterodimers with Bcl-x_L thus favoring Bax:Bax homodimer formation and apoptosis (12,20). Cell death, once suggested to be closely correlated with the percentage of Bax involved in homodimers as opposed to heterodimers with other Bcl-2 family members (12), recently has been suggested to reflect the preponderance of apoptosis-inhibiting members of the Bcl-2 family (including Bcl-x_L and Bcl-2) acting in a dominant fashion to limit the initiation of apoptosis at the mitochondria by preventing the release of cytochrome c (cyt c), apoptosis inducing factor (AIF), and other yet identified pro-apoptotic factors (14). Additionally, suppression of Bax

expression in breast tumorigenesis may reduce its inhibitory influence (acting in heterodimers with Bcl-x_L and Bcl-2) on the apoptosis-inhibitory members of the Bcl-2 family and enhance tumor development by reducing cell death (8).

High levels of Bcl-x_L expression have been reported for cuboidal epithelium and myoepithelium from breast with scattered expression reported in surrounding stromal tissues (21). Bcl-x_L overexpression, as compared to normal breast epithelium, is reported for 10% of breast carcinoma samples and has been suggested by the authors that Bcl-x_L expression might have been underestimated in that study due to the preponderance of stromal cells within the tissue studied (16). It has also been suggested that overexpression of Bcl-x_L may be correlated with estrogen-receptor (ER) negativity as Hsu et.al. have shown that high levels of Bcl-x_L and very low levels of Bcl-2 are characteristic of studied ER-negative human breast cancer cell lines as compared to ER-positive cell lines (22).

Results from rodent cell line experiments indicate that a minimum of two independent genetic events are required for transformation with the first event often providing for prolongation of cell viability or some other measure of growth advantage such that these cell populations are at increased risk for other, potentially transforming, mutations (23). Evidence for the involvement of oncogene cooperation in the development of breast cancer, resulting from transgenic mouse studies, indicates that the combination of c-Myc with either Neu/erbB2 or Ras (or both) yields increased mammary tumor incidence and decreased tumor latency (24). Results from c-Myc/Bcl-2 bitransgenic animals suggests that the rapid development of lymphoid tumors seen in these animals may be the result of Bcl-2-mediated suppression of apoptosis allowing for c-Myc-driven cellular proliferation (25,26,27). c-Myc/TGFα (transforming growth factor alpha) bitransgenic animals show a marked rise in mammary tumor incidence and diminution of tumor latencies as compared with single c-Myc or TGFα transgenic animals (2). Additional experiments with this c-Myc/TGFα model seem to imply that these gene products synergize in mouse mammary tumorigenesis with TGFα providing both an autocrine mitogenic signal and a block to c-Myc-induced apoptosis (7).

Evidence from cell lines derived from c-Myc transgenic mice indicates that epidermal growth factor (EGF) deprivation or treatment with transforming growth factor beta-1 (TGFβ1) results in a substantial decrease in Bcl-x_L mRNA and a concomitant increase in cellular death (apoptosis) (28). TGFβ1 is believed to be a negative growth regulatory molecule in mammary tissues having been shown to inhibit the growth of lobuloalveolar structures *in vivo* (29). TGFβ1 has also been associated with transformed breast epithelium and aggressive tumor phenotype is biopsy samples with these conflicting results concerning the action of TGFβ1 in the breast explained by inhibition of growth in the normal breast and early malignant tissues and involvement in accessory aspects of tumorigenesis, such as immune suppression and angiogenesis, in more advanced breast lesions (30,31).

Bcl-x_L expression has not been implicated in influencing cell growth or proliferation; therefore, it may follow that Bcl-x_L expression will not alter cyclin expression. It still has not been determined whether Bcl-x_L is involved in the

genesis of breast cancer or whether it can contribute to tumorigenesis in a synergistic fashion with c-Myc. It is possible that Bcl-x_L overexpression will block c-Myc-induced apoptosis and thereby facilitate the survival of MMECs that overexpress c-Myc counter to the potential tumor surveillance role of c-Myc-mediated apoptosis. This apoptotic blockade should then allow c-Myc to drive cell cycle progression and cellular transformation. If Bcl-x_L is demonstrated within this project to block c-Myc-induced apoptosis *in vivo* and aid in the acceleration of tumor onset, increase the incidence of mammary tumors, or enhance the aggressiveness of tumor progression, then it can be speculated that Bcl-x_L might provide a reasonable target for gene and drug directed therapies for the treatment of breast cancers. Additionally, by elucidating how specific oncogenes interact, intracellular signaling pathways can be resolved as targets for these oncogenes and specific expression patterns might prove to be prognostic clinical markers in mammary tumorigenesis.

This particular study should provide a convincing, *in vivo* method for defining a mechanism for possible c-Myc/Bcl-x_L cooperation in breast malignancies and has the potential to open up avenues of relevance to clinical assessment and management of breast cancer.

SUMMARY OF TRAINING AND RESEARCH ACCOMPLISHMENTS:

This annual summary of training and research accomplishments covers the period between August 1, 1997 and July 31, 1998 for Grant #DAMD17-97-1-7110 under the direction of principal investigator Matthew Hunter Jamerson. This twelve month period corresponds to the first twelve of the twenty months allotted for the completion of Specific Aims #1A and #1B as found within the grant's Statement of Work section and as follows:

I. *Specific Aim #1: Develop two mouse model systems for Bcl-xL/c-Myc expression and cooperation in mammary tumorigenesis.* ***Months 1-20***

A. *Specific Aim #1A: Mate MMTV-LTR/c-Myc animals with MMTV-LTR/Bcl-x_L animals, ascertain transgene expression using tail DNA biopsy, follow F1 generation animals for tumor onset and incidence, and assess tumor aggressiveness via reimplantation.*

B. *Specific Aim #1B: Develop and package Bcl-x_L retroviral vector, remove and infect MECs from 50 day old c-Myc transgenic female mice, reimplant transfected MECs into mammary fat pad, follow animals for tumor onset and incidence, and assess tumor aggressiveness via reimplantation.*

Training, Accomplishments, and Future Directions in Specific Aim #1A: Two MMTV-LTR (mouse mammary tumor virus long terminal repeat)/c-Myc males in the FVB background were obtained from the Charles River Laboratories (Wilmington, MA) in September 1998 and were used to develop a breeding colony of c-Myc mice through matings with wild-type FVB strain female animals; continued maintenance of our c-Myc colony is achieved through matings between c-Myc males and wild-type FVB strain females and is conducted under a breeding

license with DuPont Medical Products (Wilmington, DE). This particular breeding strategy is dictated by the fact that c-Myc female mice are unable to adequately nurse their offspring and subsequently these animals succumb to starvation. It had been proposed in the original grant that Southern blot analyses using a c-Myc probe and genomic DNA obtained from mouse tail biopsies would provide for the ascertainment of the transgenic status of mice; however, a more rapid and convenient polymerase chain reaction (PCR)-based strategy was employed as follows: genomic DNA is obtained from Proteinase K (GibcoBRL, Grand Island, NY)/SDS-based digestion and phenol/chloroform extraction of tail biopsy material and is utilized in a PCR reaction with two primers (MMTV-c-Myc 5′ primer as 5′-CCC AAG GCT TAA GTA AGT TTT TGG-3′ and MMTV-c-Myc 3′ primer as 5′-GGG CAT AAG CAC AGA TAA AAC ACT-3′) and PCR Supermix (GibcoBRL) with transgene positive animals (regardless of transgene zygosity) identified by a single band of approximately 880bp resolved on a 1.0% agarose gel.

Within the original submission, it had been proposed that Bcl-x_L/c-Myc bitransgenic animals would be generated using tetOP (tetracycline operator)/Bcl-x_L and MMTV/tTA (tetracycline transactivator protein) transgenic mice obtained from the laboratory of Dr. Priscilla Furth (University of Maryland) with Bcl-x_L expression being driven in a temporally-regulable manner by tetracycline administration and a geographically-regulable manner by the MMTV promoter-actuated tetracycline transactivator protein expression in mammary tissues. Subsequent, significantly delayed availability of the MMTV/tTA mice has led to the pursuit of two alternative strategies: the use of the tetTALuc (tetracycline transactivator protein with luciferase) transgenic mouse bred to the tetOP/Bcl-x_L mice and the development of an MMTV/Bcl-x_L transgenic mouse.

The tetTALuc transgenic mouse was developed by Shockett et.al. (32) as a self-inducing tetracycline-regulable system whereby the tetracycline transactivator protein is expressed under the control of a minimal human cytomegalovirus promoter (hCMV) and tetOP such that higher levels of tTA protein are induced in a wider variety of tissues that had been achieved with other tetracycline systems. Two breeding pairs of homozygous tetTALuc transgenic mice were obtained from the Jackson Laboratories (Bar Harbor, ME) in July 1998 and are currently being used to develop a purely homozygous breeding colony of these animals. For subsequent identification of the tetTALuc transgene complex in future offspring from cross-breedings, an optimized PCR-based analyses shall be performed on genomic DNA obtained from mouse tail biopsies (as per above) using a PCR reaction also containing two primers (Primer CMVF1 as 5′-TGA CCT CCA TAG AAG ACA CC-3′ and Primer TTAREV1 as 5′-ATC TCA ATG GCT AAG GCG TC-3′) and PCR Supermix (GibcoBRL) with transgene positive animals (regardless of transgene zygosity) identified by a single band of approximately 290bp resolved on a 1.0% agarose gel. Currently, initial breedings have led to the establishment of a colony of ten tetTALuc transgenic animals; further breeding is needed to increase this colony size for subsequent cross-breeding. Tetracycline-regulated expression of Bcl-x_L will be achieved through the cross-breeding of the tetTALuc transgenic mice and the tetOP/Bcl-x_L transgenic mice.

Four breeding pairs of tetOP/Bcl-x_L transgenic mice were obtained from the laboratory of Dr. Priscilla Furth (University of Maryland) in April 1998 and are currently have been bred to generate a colony of forty tetOP/Bcl-x_L animals. Identification of tetOP/Bcl-x_L transgene status has been assessed via a PCR-based approach using genomic DNA obtained from mouse tail biopsies and a PCR reaction containing two primers (Primer BCLTG5 as 5'-GCA TTC AGT GAC CTG ACA TC-3' and Primer BCLTG3 as 5'-CTG AAG AGT GAG CCC AGC AGA ACC-3') and PCR Supermix (GibcoBRL) with transgene positive animals (regardless of transgene zygosity) identified by a single band of approximately 450bp resolved on a 1.0% agarose gel. As previously mentioned, these animals will then be bred with the tetTALuc transgenic mice to develop bitransgenic mice where Bcl-x_L expression is tetracycline-regulable and subsequently bred with MMTV/c-Myc transgenic animals to generate the Bcl-x_L/c-Myc bitransgenic mice.

The second alternate strategy that is also being pursued is the generation of an MMTV/Bcl-x_L transgenic mouse. During the months of June and July 1998, I have observed and assisted in the creation of two transgenic animals and have subsequently been trained in the manipulations necessary for the development of the MMTV/Bcl-x_L transgenic animal. This valuable training included the following techniques: transgenic animal husbandry, mouse vasectomies, mouse superovulation and embryo harvest, embyro micromanipulation and transgene injection, and transgenic embryo injection into pseudopregnant, receptive female mice. Thus far, a DNA construct containing the human Bcl-x_L placed downstream of the MMTV-LTR has been generated and used to transfect MMECs to establish the potency of this construct in directing Bcl-x_L protein expression in mouse mammary cells. Initial results establish that this DNA construct is able to generate the Bcl-x_L protein as detected by Western blot analysis using a rabbit polyclonal antibody directed against Bcl-x (Transduction Labs, Lexington, KY). This experimental transfection will be repeated prior to use of this DNA construct (linearized by SalI and StuI restriction endonucleolysis) in microinjection of FVB and C57B strain mouse embryos for generation of the MMTV/Bcl-x_L transgenic mice.

The final issue that I am currently addressing with respect to Specific Aim #1A involves the cross-breeding strategy to be employed for the generation of the Bcl-x_L/c-Myc bitransgenic mice. Generation of this bitransgenic mice from cross-breeding between the MMTV/c-Myc mice and the in-development MMTV/Bcl-x_L mice will prove rather straightforward requiring only that the parental animals used possess one copy of their specific transgene (this breeding does not require attention to P1 generation transgene zygosity status - a heterozygous c-Myc animal bred to a heterozygous Bcl-x_L animal has the chance to produce bitransgenic F1 offspring). Generation of a Bcl-x_L/c-Myc bitransgenic animal using the MMTV/c-Myc, tetOP/Bcl-x_L, and tetTALuc transgenic mice will prove more difficult and will require attention to transgene zygosity status. The PCR-based strategies that are currently being used to determine the transgene status of these animals are non-discriminatory between the heterozygote and homozygote status and therefore only reflect the presence or absence of the transgene. To address this issue, I have designed a PCR capture strategy to identify the mouse genomic sequences both upstream and downstream from the MMTV/c-Myc and tetOP/Bcl-x_L transgenes.

This PCR capture method will be carried out as follows: first, a biotinylated capture primers designed to hybridize to a 5′ portion of the MMTV-LTR (Myc Capture Primer as 5′-bioGAA TAT GTC TTT GTC TGA TGG GCT C-3′) will be used, in conjunction with streptavidin-conjugated paramagnetic beads (Promega, Madison, WI), to pull down transgene DNA and flanking mouse genomic DNA (that has been cut with a restriction endonuclease); second, the captured DNA will be extended with polymerase and then linked with a blunted-ended linker/primer pair; third, a secondary restriction endonuclease will be utilized to squelch contamination by tandem-arrayed transgene sequence material that might obfuscate identification of mouse genomic DNA (both of these transgenic animals, as is common with all generated by this method, typically contain the transgenetic material inserted at a single locus in multiple, tandemly-arrayed copies); and fourth, the sequence of the captured mouse genomic DNA will be evaluated using both the linker primer and an additional PCR primer (5′-GGA AAC AAC CCC TTG GCT GC-3′). Once these yet unidentified flanking sequences have been defined, they will facilitate the development of a tertiary PCR primer that can then be used with the two aformentioned c-Myc and Bcl-x_L primers to resolve the transgene zygosity status of both c-Myc and Bcl-x_L animals.

<u>Training, Accomplishments, and Future Directions in Specific Aim #1B</u>: Within the original submission, it had been proposed that a Bcl-x_L retrovirus be constructed and utilized to infect MMECs explanted and cultured from MMTV/c-Myc transgenic mice. The initial choice of retrovirus proposed was the pNO4 expression system containing the Moloney Sarcoma Virus (MCV) LTR, a neomycin resistance cassette, and a CMV promoter to drive expression of the Bcl-x_L cDNA inserted into the retrovirus. I have subsequently decided to approach this specific aim using the LNSX retrovirus created by Dusty Miller (33) since this particular system uses an simian virus 40 (SV40) promoter to drive insert expression, can be developed to a much higher titer of infective retrovirus, and has been constructed to prevent the production of interfering helper viruses. The pLNSX retroviral construct was received in July 1998 and has been used to transform competent JM109 bacterial cells to produce large quantities of DNA for further characterization and subsequent generation of the Bcl-x_L-expressing retrovirus. Also completed are the restriction mapping of the pLNSX and the preparation of the human Bcl-x_L cDNA (with digestion by XbaI and ClaI to allow for compatible end generation to the retroviral AvrII and ClaI sites in the multiple cloning region) that will be ligated into the LNSX construct to generate the retrovirus of interest.

Once fully generated and titered, this particular retrovirus will then be used *in vitro* to infect MMECs obtained from c-Myc transgenic mice. These infected MMECs will then be selected for using the neomycin resistance property of the LNSX retrovirus, expanded in culture, and reinjected into cleared mammary fat pads of littermate animals (littermates to the animal from which the infected MMECs were obtained) to reconstitute mammary glands. These animals will then be followed as are the fully bitransgenic animals to be generated through Specific Aim #1A.

Note 1: As a secondary, complementary project to the development and assessment of the Bcl-x_L/c-Myc transgenic mouse mammary cancer model, I am currently developing a Bax-knockout/c-Myc transgenic mouse model for mammary cancer. Currently, a breeding colony of approximately 60 Bax knockout animals has been generated that will next be used for cross-breeding with male MMTV/c-Myc transgenic mice to generate animals that possess both the c-Myc transgene and single and double allele knockouts of the endogenous, tumor-suppressor-like Bax gene. The rationale behind this study comes from the fact that Bax is known to interact with anti-apoptotic proteins, such as Bcl-x_L, in controlling cell death(12), Bax protein expression has been shown to be significantly reduced or altogether absent in breast tumors and cell lines(34), reduced Bax expression has been correlated with both treatment failure and shorter survival(35), Bax has been shown to possess a tumor-suppressor type activity when transfected into breast cancer cell lines via reduction of proliferation and increased apoptosis(36), and Bax protein is thought to be induced by Myc expression (due to a myc-responsive element) and to be involved in Myc-driven apoptosis (37,7). For these reasons, it seems that the development of the Bax-knockout/c-Myc transgenic mouse tumor model will prove complementary to the Bcl-x_L/c-Myc bitransgenic tumor model.

Note 2: The principal investigator, Matthew Hunter Jamerson, was involved in the completion of graduate coursework during the period stretching from August 1997 through May 1998 while conducting the research described in this annual summary for Grant #DAMD17-97-1-7110.

CONCLUSIONS:

During the first year of work on Grant #DAMD17-97-1-7110, a number of significant goals have been accomplished in the development of the proposed transgenic mouse breast cancer models to include the establishment of animal breeding colonies for the major transgenic animals involved in the study, the development and optimization of strategies for the identification of the transgenic status of these animals, and the refinement of strategies to be employed in assessing transgene zygosity and retroviral vector construction. Additionally significant have been the numerous research-oriented skills that this principal investigator has acquired during the course of work on this grant to include the following: animal breeding and husbandry, animal surgeries, mouse embryo harvesting and DNA microinjection, transgenic animal generation, numerous DNA manipulatory procedures including restriction analyses and construct generation, primer selection and PCR, PCR-based capture methods for assessment of flanking genetic sequences of known sequences, cell and tissue culture, Western blot analysis, bacterial transformation and mammalian cell transfection. Currently, work on this project is approaching the point of generation of the first sets of bitransgenic animals for assessment of these animals as mammary cancer models as set forth in the grant proposal.

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